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Published in:
Research in Veterinary Science

DOI:
[10.1016/j.rvsc.2019.10.015](https://doi.org/10.1016/j.rvsc.2019.10.015)

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Ryt-Hansen, P., Larsen, I., Kristensen, C. S., Krog, J. S., & Larsen, L. E. (2019). Limited impact of influenza A virus vaccination of piglets in an enzootic infected sow herd. *Research in Veterinary Science*, 127, 47-56.
<https://doi.org/10.1016/j.rvsc.2019.10.015>



Limited impact of influenza A virus vaccination of piglets in an enzootic infected sow herd

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ARTICLE INFO

Keywords:

Influenza A virus
Swine influenza
Piglet vaccination
Influenza vaccination
Recurrent shedding
Reinfection

ABSTRACT

Recent studies have questioned the effect of maternal derived antibodies (MDAs) to protect piglets against infection with influenza A virus (IAV). The lack of protection against IAV infections provided by MDAs has encouraged alternative vaccination strategies targeting young piglets in an attempt to stimulate an early antibody response. There is a lack of studies documenting the efficacy of piglet vaccination. In the present study, we monitored a group of vaccinated and non-vaccinated piglets in a Danish sow herd that initiated piglet vaccination with ¼ dose of an inactivated swine influenza vaccine at the time of castration (day 3–4). A total of 160 piglets from 11 sows were included and either vaccinated with 0.5 mL inactivated swine influenza vaccine or sham-vaccinated. From week 0 until week 6, all included piglets were clinically examined and nasal swabbed once per week and weighed at weeks 0, 3 and 6. Blood samples were collected from sows at week 0 and from piglets at week 3. Vaccination of piglets had limited effect on clinical signs, body weight, antibody development and viral shedding, within the first 6 weeks of life. At least 50% of all pigs of each treatment group tested positive for IAV at week 2, and very early onset of IAV shedding was observed. In total, 18 pigs were IAV positive in nasal swabs for more than one consecutive sampling time indicating prolonged shedding and 14 pigs were IAV positive with negative samplings in between indicating re-infection with the same IAV strain.

1. Introduction

Influenza A virus (IAV) infection in pigs can lead to clinical signs of respiratory disease and compromised animal welfare, increased use of antibiotics and negative impact on the productivity (Fablet et al., 2012; Opriessnig et al., 2011; Vincent et al., 2014). Furthermore IAV is a zoonotic disease, which leaves swine herds as a reservoir for possible future human IAV pandemics (Garten, 2009; Torremorell et al., 2012). These factors emphasize the need for an optimal control strategy to minimize the number of IAV positive pigs and herds. Recent studies documented changes in the IAV dynamics, and it is now clear that epizootic infections, in most cases, will establish enzootic status in affected herds (Cador et al., 2017; Cappuccio et al., 2017; Diaz et al., 2017b; Ferreira et al., 2017a; Loeffen et al., 2009; Pitzer et al., 2016; Rose et al., 2013; Simon-Grifé et al., 2012). Several studies investigated the effects and benefits of maternally derived antibodies (MDAs) in protection against IAV infection in the piglets, and these studies showed that MDAs do not provide full protection against IAV infection and clinical signs (Allerson et al., 2014; Cador

et al., 2016c, 2016b; Corzo et al., 2014; Diaz et al., 2017a; Markowska-Daniel et al., 2011; Rose et al., 2013; Simon-Grifé et al., 2012). Moreover, presence of MDAs at the time of primary infection may impair an active humoral response making the piglet susceptible for a reinfection even with the same strain (Cador et al., 2016a; Deblanc et al., 2018; Loeffen et al., 2003). A previous study revealed that IAV infection can occur as early as 3 days of age despite the presence of MDA and IAV is highly prevalent in both the farrowing unit and in nursery pigs early after weaning (Corzo et al., 2014; Ferreira et al., 2017b; Rose et al., 2013; Ryt-Hansen et al., 2019; Simon-Grifé et al., 2012). In Europe, there is no vaccine licensed for use in pigs below 8 weeks of age and sow vaccination strategies (mass sow vaccination/pre-farrow) are widely applied for limiting the clinical impact of IAV in piglets by ensuring uptake of MDAs in the piglets (Rajao et al., 2014). However, the protection obtained through MDAs can, as mentioned above, be sub-optimal and therefore different approaches to control IAV in young piglets e.g. by vaccination are needed. The aim of the present field study was therefore to monitor clinical signs, weight, antibody development and viral shedding in piglets in a Danish herd that vaccinated piglets

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<https://doi.org/10.1016/j.rvsc.2019.10.015>

Received 9 August 2019; Received in revised form 22 October 2019; Accepted 23 October 2019

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with a ¼ dose (0.5 mL) of an inactivated swine influenza vaccine (RespiPorc FLU3, IDT Biologika) per piglet at the time of castration.

2. Materials and methods

2.1. The herd

This case took place in a Danish herd with 900 sows and 3500 pen places for nursery pigs. The herd was according to the Danish Specific Pathogen Free program (Svineproduktion, 2019) declared free from Porcine Reproductive & Respiratory Syndrome Porcine virus (PRRSv). No strict all in/all out strategy was performed in any of the units, and stables were only cleaned and disinfected with hydrated lime between the different batches of sows in the farrowing unit. No quarantine stable was present for incoming gilts. In the farrowing unit, a high number of nursing sows were used and the piglets were mingled more than once. No vaccination against IAV had been used previously in the herd. All piglets were vaccinated against *Mycoplasma hyopneumoniae* at day 4. Recurrent problems with respiratory disease were seen and the herd had previously tested positive for influenza A virus (subtype H1avN2).

2.2. Study design

A total of 160 piglets from 11 sows that farrowed on the same day were selected for the study. To assure colostrum intake, no litter equalization was allowed before castration at day 4, where all piglets were ear tagged with consecutive numbers. All movements were recorded. Pigs with an odd ear tag number were injected intramuscularly in the neck with 0.5 mL RespiPorc FLU3 (VAC), corresponding to a quarter of a dose and piglets with an even ear tag number were injected with 0.5 mL of physiological saline, 9 mg/mL (control).

2.3. Data sampling

Clinical examination of all ear tagged pigs was performed weekly and included registration of nasal discharge, conjunctivitis, lacrimation and fecal soiling. In addition, the pigs were evaluated for body score and “other diseases” including clinical signs of lameness, wounds, eczema, umbilical hernia and CNS symptoms. Rectal temperature was measured at the time of vaccination and one-day post vaccination on all ear tagged pigs.

Weighing of all pigs was performed at weeks 0, 3 and 6 on a piglet scale approved for pigs weighing between 200 g and 30 kg (Bjerringbro Vægte Aps, Denmark).

Blood samples were drawn from *vena cava cranialis* of all ear tagged pigs at week 3 and from *vena jugularis* of the sows at week 0. All blood samples were collected in a vacutainer serum tube (Becton Dickinson, Denmark). The blood samples were kept at 5 °C for a maximum of 2 days, until they were centrifuged at 3000 rpm for 10 min and the resulting serum was immediately analyzed.

Nasal swabs were collected from all piglets at the time of vaccination (week 0) and weekly thereafter until week 6. As not all the piglets were weaned at the same age, sampling during week 4 was done either in the farrowing unit (W4F) or in nursery unit (W4N). The swab, a small rayon swab (Medical Wire, UK), was introduced in both nostrils where it was turned 360 degrees. Afterwards, the swab was transferred into the Sigma Virocult media (Medical Wire, UK), and kept at 2–8 °C for a maximum of 2 days.

2.4. Laboratory analyses

2.4.1. Serology

The blood samples from sows and pigs were tested in a blocking ELISA that detects antibodies targeting the NP gene, which is conserved among the different Influenza A subtypes (IDEXX; Influenza A Ab Test; IDEXX Laboratories, Inc.)

2.4.2. Pooling of samples and RNA extraction

The Sigma Virocult media (MWE, England) containing the swab was vortexed and poured into a 1.5 mL tube (Eppendorf), wherefrom 100 µL were withdrawn for the pool. Five nasal swabs representing five pigs were pooled in same tube. Vaccinated and control animals were pooled in separate tubes. The pool was mixed and centrifuged and subsequently 200 µL was withdrawn and transferred to a tube containing 400 µL RLT-buffer (QIAGEN, Copenhagen, Denmark) containing 2-Mercaptoethanol (Merck, Darmstadt, Germany). The RNA was extracted from the sample using the RNeasy mini kit (QIAGEN,) automated on the QIAcube (Qiagen, Hilden, Germany) using the large sample protocol version 2.

2.4.3. Real time RT-PCRs

A previously published semi-quantitative real time RT-PCR assay targeting the matrix gene of all IAVs (Nagy et al., 2010) was utilized to determine if a pool was positive for influenza A virus. Briefly, the OneStep RT-PCR kit (QIAGEN) was used with the published primers, and all PCR reactions were run on the Rotor-Gene Q (QIAGEN) using the following program: 50 °C, 30 min; 95 °C, 15 min; cycling 45 × (95 °C, 10 s, 60 °C 20 s, 64 °C 1 s, 68 °C 1 s, 72 °C 30 s). All PCR reactions were run in duplicates, and the sample was only considered positive if both duplicates gave a Ct value. A pool was considered positive if it had a Ct value < 36. If a pool tested positive, the RNA was extracted from the individual samples of the pool, using the same method as described above. The RNA was then subjected to the same semi-quantitative real time RT-PCR assay as described above to determine which of the individual pigs that was positive for IAV. All positive individual samples with a Ct value < 31 were then tested in a multiplex real time RT-PCR assay for the determination of the Influenza A subtype as previously described (Ryt-Hansen et al., 2019).

2.4.4. Viral isolation and NGS

The nasal swab with the lowest Ct value, was selected for viral isolation in MDCK cells. The MDCK cells were incubated at 37 °C in a 5% CO₂ atmosphere in a viral growth media containing Minimum Essential Medium Eagle (MEM) (Gibco, Carlsbad, CA, USA), 5% inactivated fetal calf serum (FCS), Non-essential amino acids (NEAA), 2 mM L-glutamine and Penicillin-streptomycin. The nasal swab was subjected to sterile filtration using a 0.45 µm Millex-HP Millipore filter (Merck, Germany), and 200 µL was then used for inoculation of cells along with media containing MEM, NEAA, 2 mM L-glutamine, Penicillin-streptomycin and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated Trypsin. After 24 h, the supernatant was harvested. The RNA was extracted from the supernatant as described earlier, and the RNA used as a template for a modified version of the one-tube PCR protocol as previous described (Kai Lee, 2013). In short, the primers MBTuni-12 and MBT-uni13 were used together with the SuperScript III RT/Platinum Taq High Fidelity kit (Invitrogen, Denmark), and run on the T3 thermocycler (Biometra, Denmark) with the following conditions: 42 °C, 60 min, 94 °C, 2 min, 5 × (94 °C, 30 s – 45 °C, 30 s – 68 °C, 180 s), 31 × (94 °C, 30 s – 57 °C, 30 s – 68 °C, 180 s) and 68 °C, 7 min. The PCR products were visualized with UV-light on a 0.8% agarose E-gel (Thermo Fisher Scientific) and then purified with the High Pure PCR Product Purification Kit (Roche, Denmark). Thereafter, the sample was sent for whole genome sequencing on the Illumina MiSeq platform at Statens Serum Institut (Copenhagen, Denmark).

2.4.5. Conventional PCR and Sanger sequencing

Samples from pigs either testing positive for IAV for more than two consecutive sampling times, and samples from pigs testing positive for IAV at non-consecutive sampling times with minimum two negative sampling time points in between, were chosen for Sanger sequencing of the hemagglutinin (HA) and neuraminidase (NA) genes. The primers listed in Table 1 were used for the PCR along with the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kit

Table 1
Primers for conventional PCR of the HA and NA genes.

	Sequence
HA-gene	
Forward primer (pQE-HA-S-F)	5'- CGG ATA ACA ATT TCA CAC AGA GCA AAA GCA GGG GAW AAT W -3'
Reverse primer (pQE-HA-R)	5'- GTT CTG AGG TCA TTA CTG GAG TAG AAA CAA GGG TGT TTT -3'
NA-gene	
Forward primer (pQE-NA-F)	5'- CGG ATA ACA ATT TCA CAC AGA GCA AAA GCA GGA GT -3'
Reverse primer (pQE-NA-R)	5'- GTT CTG AGG TCA TTA CTG GAG TAG AAA CAA GGA GTT TTT T -3'

W = A or T according to the IUPAC nucleotide code.

(Thermofisher Scientific, Copenhagen, Denmark). The PCR was run on the T3 thermocycler (Biometra, Fredensborg, Denmark) with the following program for the HA gene: 55 °C for 30 min, 94 °C for 2 min, 4 × (94 °C for 30 s, 55 °C for 30 s and 68 °C for 180 s), 41 × (94 °C for 30 s and 68 °C for 210 s) and 68 °C for 10 min and the following program for the NA gene: 54 °C for 30 min, 94 °C for 2 min, 4 × (94 °C for 30 s, 58 °C for 30 s and 68 °C for 180 s), 41 × (94 °C for 30 s and 68 °C for 210 s) and 68 °C for 10 min. The PCR product was thereafter visualized on a gel and purified, as described above for the NGS sequencing. The purified PCR products were sent for Sanger sequencing with the PCR primers at LGC Genomics (Berlin, Germany).

2.4.6. Generation of consensus sequences

The whole genome sequencing data was imported into the program CLC genomics Workbench version 11.0.1. The reads were paired and trimmed, and then mapped against a set of 22 reference segments, covering all IAV segments known to circulate in Denmark. The mapping result with the highest number of reads and the correct consensus length was used to extract a consensus sequence for each of the eight genome segments. The HA consensus sequence was translated into amino acids. The Sanger sequencing data was imported into CLC main workbench version 8, and were assembled against reference sequences of the HA and NA genes. The reference sequences used were sequences with the highest scoring sequence identity to the forward and reverse reads using the function “BLAST against NCBI”. The sequences were then manually proofread and the consensus sequence of each sample was extracted and translated to amino acids.

2.4.7. Analysis of the consensus sequences

To confirm the subtype(s) of the IAV strain circulating in the herd, all HA and NA genes were aligned with contemporary HA and NA sequences obtained in the Danish annual swine IAV surveillance and a neighbor-joining tree was constructed. Thereafter, all HA and NA nucleotide and amino acid consensus sequences were aligned using the MUSCLE algorithm (Edgar, 2004) and compared using the “pairwise comparison” tool in CLC main workbench version 8. A similar, alignment and comparison was also performed for both the HA and NA consensus sequences of the herd strain against the respective vaccine consensus HA and NA sequence. The HA of A/swine/Haselünne_IDT_2617_2003 (H1N1) with accession number: GQ161124 (nucleotides) and ACR39185 (amino acids) was used for the HA alignment and the NA of A/swine/Bakum/IDT1769/2003 (H3N2) with accession number GQ161100 (nucleotides) and ACR39300 (amino acids) was used for the NA alignment. To investigate if amino acid differences between the herd strains and the vaccine strain were in any of the known antigenic sites of the HA protein (Caton et al., 1982; Manicassamy et al., 2010; Rudneva et al., 2012; Yang et al., 2012), the location of the sites were annotated to each amino acid sequence in the alignment. Finally, to reveal any sequence differences between the HA sequence obtained from the same pig at different sampling times (prolonged or recurrent shedders) the HA consensus sequences of the same pig were individually aligned and compared as described above, and the same antigenic sites were annotated to the amino acid sequences. The consensus sequences of each internal gene (M, NS, NP, PA, PB1 and PB2) was investigated for the closest sequence

identity in GenBank using the function “BLAST against NCBI” to determine if they were of avian or pandemic (A(H1N1)pdm09) origin. In addition, to confirm the BLAST results, all the internal genes were separately aligned with contemporary internal gene sequences obtained in the Danish swine IAV surveillance program and a neighbor-joining tree was constructed.

2.5. Statistical analyses

Sample size calculations were based on an average weight at weaning of 6.0 kg with a standard deviation of 1.0 kg. With a significance of 5%, a power of 80%, the required samples size of a two-sided test, was 63 piglets per group to prove a difference in weight at weaning of 0.5 kg statistically significant (Houe et al., 2004).

For comparison of clinical signs (lacrimation, nasal discharge or conjunctivitis) between VAC and control pigs a Pearson's Chi-squared Test was performed.

For an overall statistical comparison of means from normally distributed data (e.g. mean weight between VAC and control pigs), a Student's *t*-Test was performed. In addition, the pigs were divided into two groups based on the average Ct value of all positive nasal swabs during the entire study period. Pigs with a Ct value higher than the average Ct value were defined as “low infection level”, and pigs with a lower or equal Ct value than the average were defined as “high infection level”. Since the two treatment groups were equally distributed according to infection level the analysis of “low infection level”/“high infection level” were done without including vaccination status. The relationship between infection level (low/high) and the mean weight were analyzed with a Student's *t*-Test and clinical signs with a Pearson's Chi-squared Test. Furthermore, the impact of transfer of pigs between sows on clinical signs, infection level and weight were analyzed with Pearson's Chi-squared Test and a Student's *t*-Test, respectively. The antibody status of the sows (positive/negative) at week 0 was tested for correlation to the number of virus positive and negative piglets at the different sampling times, using a Pearson's Chi-squared Test.

Each time point (0–6) was analyzed both separately and in total. Statistical analyses were computed in R version 3.5.1 (R Core Team 2018).

3. Results

A total of 160 piglets from 11 sows were included in the study at week 0, of which 80 received vaccination with RespiPorc FLU3 and 80 were sham vaccinated with physiological saline, 9 mg/mL (control). Between week 0 and week 2, 52 piglets died due to diarrhea. By the end of the study, 102 piglets were still alive (Table 2).

3.1. Clinical signs

One day after vaccination, none of the pigs experienced pyrexia. The mean rectal temperature was 38.8 °C (sd = 0.6) in the vaccinated group and 38.8 (sd = 0.6) in the control group.

Results of the weekly clinical examinations of the pigs in both treatments groups independently of their IAV status are shown in

Table 2

Overview of total number of pigs, % IAV positive pigs, mean Ct values, infection level and clinical signs between the two groups (VAC and control) over each of the 6 sampling times and accumulated as a total.

Age (week)	0 (vaccination)	1	2	3	4F	4 N	5	6	Total
Stable unit	Farrow	Farrow	Farrow	Farrow	Farrow	Nursery	Nursery	Nursery	
Number of piglets									
Total	160	123	108	104	53	49	101	102	800
VAC	80	61	56	55	28	26	53	54	413
Control	80	62	52	49	25	23	48	48	387
Influenza virus positive pigs, <i>n</i> (%)									246
VAC	3 (3.8)	10 (16.4)	30 (53.6)	11 (20.0)	17 (63.0)	7 (26.9)	23 (43.4)	20 (37.0)	121
Control	5 (6.3)	9 (14.5)	26 (50.0)	10 (20.4)	14 (56.0)	17 (73.9)	28 (58.3)	16 (33.3)	125
Ct among virus positive pigs, mean									
VAC	33.90	30.26	28.15	28.56	28.15	26.30	30.37	30.57	29.2
Control	30.11	30.86	26.39	26.84	28.47	30.90	30.05	30.32	29.1
Influenza virus, pigs with high shedding (Ct < 29.19), <i>n</i> (%)									
VAC	0 (0)	2 (3.2)	14 (25.0)	3 (5.4)	5 (23.8)	4 (15.4)	7 (13.2)	5 (9.3)	40 (9.7)
Control	2 (2.5)	1 (1.6)	15 (28.8)	7 (14.3)	6 (33.3)	3 (13.0)	8 (16.7)	5 (10.4)	47 (12.1)
Clinical signs, <i>n</i> (%)									
Lacrimation									25 (3.1)
VAC	4 (5.0)	1 (1.6)	0 (0.0)	0 (0.0)	2 (7.1)	0 (0.0)	1 (1.9)	0 (0.0)	8 (1.9)
Control	10 (12.5)	2 (3.2)	1 (1.9)	2 (4.1)	1 (4.0)	0 (0.0)	1 (2.0)	0 (0.0)	17 (4.4)
Nasal discharge									380 (47.6)
VAC	8 (10.0)	33 (54.1)	21 (37.5)	39 (70.9)	23 (82.1)	3 (11.5)	28 (52.8)	49 (90.7)	204 (49.4)
Control	11 (13.8)	22 (35.5)	12 (23.1)	33 (67.3)	22 (88.0)	10 (43.5)	34 (69.4)	32 (69.6)	176 (45.6)
Conjunctivitis									289 (36.2)
VAC	14 (17.5)	22 (36.1)	30 (53.6)	34 (61.8)	17 (60.7)	12 (46.2)	9 (17.0)	17 (31.5)	155 (37.5)
Control	24 (30.0)	25 (40.3)	30 (57.7)	13 (26.5)	10 (40.0)	8 (34.8)	12 (24.5)	12 (26.1)	134 (34.7)
Pigs with either lacrimation, nasal discharge or conjunctivitis									540 (67.6)
VAC	24 (30.0)	48 (78.7)	39 (69.6)	46 (83.6)	23 (82.1)	14 (53.8)	31 (58.5)	50 (92.6)	275 (66.6)
Control	37 (46.3)	42 (67.7)	38 (73.1)	35 (71.4)	22 (88.0)	16 (69.6)	39 (79.6)	36 (78.3)	265 (68.6)
Poor body score									294 (36.8)
VAC	41 (51.2)	23 (37.7)	14 (25.0)	15 (27.3)	6 (22.2)	9 (34.6)	13 (24.5)	17 (31.5)	138 (20.3)
Control	43 (53.8)	26 (41.9)	12 (23.1)	13 (26.5)	8 (32.0)	11 (47.8)	23 (46.9)	20 (42.6)	156 (22.2)
Fecal soiling									170 (21.3)
VAC	24 (30.0)	17 (27.9)	10 (17.9)	5 (12.7)	1 (3.6)	2 (7.7)	17 (32.1)	6 (11.1)	84 (20.3)
Control	24 (30.0)	17 (27.4)	9 (17.3)	7 (10.2)	2 (8.0)	2 (8.7)	18 (36.7)	9 (19.6)	86 (22.3)

The results are listed as pig age group (in weeks) along with the stable unit in which the pigs were present when sampled. The pigs are further divided into treatment group (VAC vs Control) when tested against the different parameters. Statistically significantly different results ($p < .05$) between the VAC/Control groups ($p < .05$) are highlighted in bold.

Table 2. When considering the weekly clinical signs of the pigs in both treatments groups, both positive and negative correlations were discovered, and no significant difference was consistent over more than one sampling time (Table 2).

The total number of pigs showing any of the clinical signs over the whole study period (sum of all weeks) was compared to the treatment group, stable unit, IAV presence and level, body condition score, transfer between sows and antibody status of the sows. No significant difference was observed in any of the clinical signs between the treatment groups. However, a strong significant correlation ($p < .001$) was found between the presence of nasal discharge and the presence of IAV in the nasal swabs, independently of the treatment group. The presence of at least one of the three clinical signs of respiratory disease; nasal discharge, conjunctivitis or lacrimation also had a significant correlation to the presence of IAV independently of the treatment group ($p \leq .05$). A slightly weaker and non-significant correlation ($p = .078$) between conjunctivitis and the presence of IAV was also found, independently of the treatment group. No correlation between lacrimation and the presence of IAV was found, and no correlation was found

between the infection level (high or low) and the clinical signs. Interestingly, the transfer of pigs between sows had both a negative impact of the body condition score, fecal soiling and increased the number of pigs showing one of the three clinical signs correlated to respiratory disease (Supplementary Table 1).

The summed numbers of each of the clinical signs over the whole study period of the virus positive pigs exclusively are listed in Table 3. No significant differences were found when comparing the degree of each of the clinical signs between the virus positive vaccinated pigs and the virus positive control pigs.

3.2. Body weight

The mean body weight of the vaccinated group were 1.34 kg (sd = 0.4) at week 0, 5.19 kg (sd = 1.4) at week 3 and 8.25 kg (sd = 1.9) at week 6, whereas the mean weight of the control group were 1.32 kg (sd = 0.4) at week 0, 5.10 kg (sd = 1.5) at week 3 and 7.83 kg (sd = 2.4) at week 6 (Fig. 1). No significant differences in average body weights were observed between the vaccinated pigs and

Table 3

Prevalence of clinical signs in IAV positive pigs of the two treatments groups.

	Lacrimation	Nasal discharge	Conjunctivitis	Respiratory signs ≥ 1	Poor body score	Fecal soiling
Observations in total, <i>n</i>	799	799	799	799	799	799
Cases, <i>n</i> (% of total)	24 (3.1%)	376 (47.6%)	286 (36.2%)	537 (67.6%)	294 (37.0%)	170 (21.3%)
Virus positive, <i>n</i>	4	142	100	182	79	55
VAC, <i>n</i>	1 (25%)	72 (50.7%)	57 (57%)	89 (49.9%)	31 (39.2%)	22 (40%)
Control, <i>n</i>	3 (75%)	70 (49.3%)	43 (43%)	93 (51%)	48 (60.8%)	33 (60%)

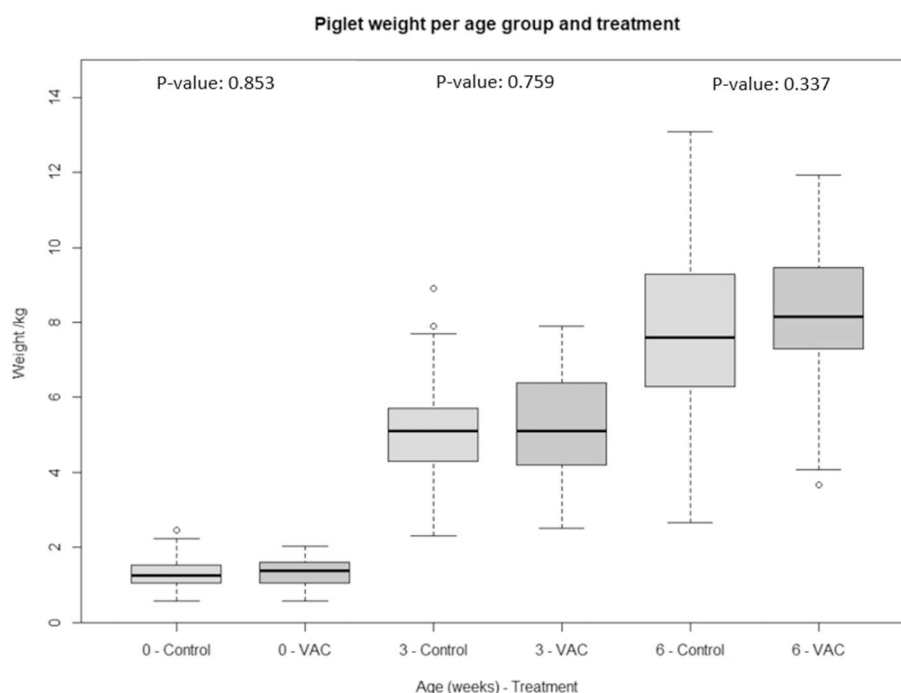


Fig. 1. Boxplot showing the weight of the pigs of the two groups (control vs vaccine) at the three weighing times (week 0, 3 and 6).

control pigs at any of the three sampling times. However, a significant lower bodyweight was revealed in the IAV positive pigs at week 6 compared to IAV negative pigs, which weighed 1.7 kg more. Furthermore, the infection level, defined as higher or lower than the average Ct value (29.2, described in the section “Level of virus in nasal swabs”) also had a significant impact on the body weight at week 6, as the pigs with a “high infection level” weighed 1.47 kg less than the pigs with “low infection level” (Supplementary Table 2).

3.3. Serology

The blood samples obtained from the sows at week 0 revealed that the majority of the sows (9/11) were positive for antibodies against IAV.

There was no significant difference in the percentage of seropositive piglets at week 3 between the two treatment groups in that 80% of the controls and 73% of the vaccinated pigs were antibody positive and the average OD-ratio did not differ significantly between the two treatment groups (control: 0.48 and vaccinated: 0.49) (data not shown).

3.4. Prevalence of IAV

The percentage of piglets testing positive for IAV in nasal swabs over the study period is shown in Fig. 2. Already at the time of castration (day 3–4), eight piglets tested positive for IAV in nasal swabs. The prevalence of virus positive piglets then increased markedly and at week 2 approx. 50% of all pigs of both groups tested positive for IAV in the nasal swabs. At week 3, a decrease in the prevalence was observed and then the prevalence increased again after weaning (week 4), where most of the pigs were mixed in the nursery stables. The only significant difference in the number of IAV positive pigs between the two groups was observed at the first sampling in the nursery unit (W4N) where a significant higher number of IAV positive piglets were present in the control group compared to the vaccinated group. The results revealed that all but one pig, which survived throughout the study period tested positive for IAV at some point during the study, resulting in no overall difference in the total number of infected pigs between the two treatment groups (Supplementary Table 3).

The percentage of virus positive pigs originating from either an antibody positive or negative sow is shown in Fig. 3. Although the number of seronegative sows was very low (2/11 sows), there was a significant higher number of pigs from antibody negative sows that became infected during the first three sampling times at weeks 0, 1 and 2 compared to piglets from the seropositive sows. In contrast, the prevalence of positive piglets was the same at weeks 3–5 irrespectively of the antibody status of the sow, but, interestingly, significantly more pigs from the antibody positive sows were infected at week 6. However, no significant difference was observed in the total percentage of IAV positive piglets between the two treatment groups.

3.5. Duration of shedding time and virus subtype

In total, seven pigs from the vaccinated group and eleven pigs from the control group tested positive for IAV in the nasal swabs over three consecutive sampling times and one piglet from the control group

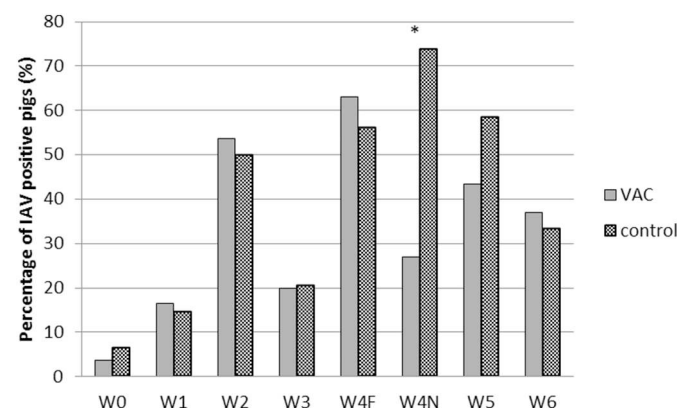


Fig. 2. Prevalence of IAV in nasal swabs of the control and vaccinated (VAC) groups over the different sampling times (W0–W6).

W4F: samples taken in week 4 in the farrowing unit. W4N: samples taken in week 4 in the nursery unit.

*Indicates a significant difference ($p < .05$) between the two groups.

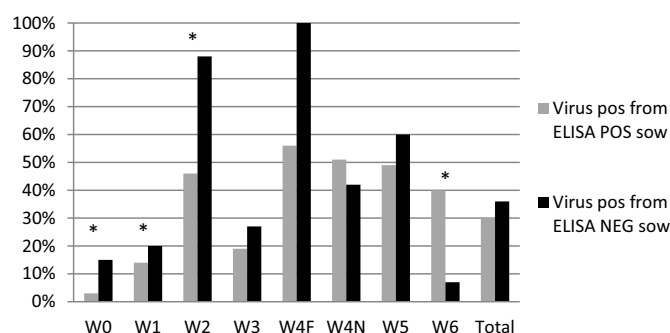


Fig. 3. Prevalence of IAV positive pigs from seropositive or seronegative sows over the different sampling times (W0–W6).

W4F: samples taken in week 4 in the farrowing unit. W4N: samples taken in week 4 in the nursery unit.

*Indicates a significant difference ($p < .05$) between the two groups.

tested positive for IAV over four consecutive sampling points. We defined these pigs as “prolonged shedders”. Furthermore, 14 pigs tested positive for IAV at nonconsecutive sampling points separated by two or three sampling times. This number included eleven nonconsecutive shedders from the vaccinated group and three from the control group (Supplementary Table 3). We defined these pigs as “recurrent shedders”.

All samples of the study with a Ct value < 31 ($n = 45$) were subtyped by a multiplex RT-real time PCR and revealed the herd subtype to be of H1avN2sw origin (data not shown).

3.6. Level of virus in nasal swabs

Fig. 4 list the viral load (estimated as average Ct values) in the nasal swabs of the positive pigs from both treatment groups during the study period. The only significant difference in viral load was in the pigs in the nursery unit at week 4. Unexpectedly, the vaccinated pigs had a higher level of viral shedding compared to unvaccinated controls.

The range in average Ct values of the two treatment groups was between 26.3 and 33.9 (Table 2). The average Ct value based on all the results of all piglets at all sampling times was 29.2, and this value was, as previously mentioned, used as a cut off for defining the pigs as having a “high infection level” or a “low infection level”. When using this definition of infection level it was evident that there was no significant difference between the numbers of pigs with high and low infection level according to vaccination status (Table 2).

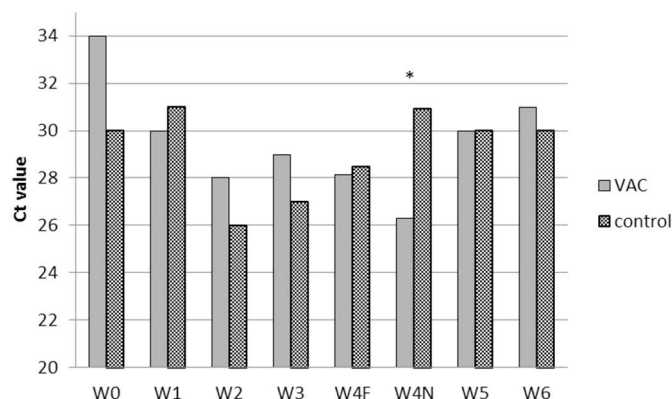


Fig. 4. Average Ct values of the IAV positive pigs of the control and vaccinated (VAC) groups over the different sampling times (W0–W6).

W4F: samples taken in week 4 in the farrowing unit. W4N: samples taken in week 4 in the nursery unit.

*Indicates a significant difference ($p < .05$) between the two groups.

3.7. Genetic characterization of the herd IAV strain

Full genome sequences of all eight segments of the herd strain were obtained. The results revealed that the sample was of the H1avN2sw subtype, which agreed with the results of the multiplex RT-real time PCR. Moreover, the results of the BLAST revealed that the M, NP, PA, PB1 and PB2 genes were of the pandemic A(H1N1)pdm09 origin, whereas the NS gene was of avian origin. The full genome sequences from this study are available in the NCBI Genbank with the following accession numbers: MN249749–MN249756. The Sanger sequencing results derived from a total of 21 samples confirmed the HA and NA subtype and thereby documented that only one strain was circulating in the herd throughout the study period. The HA sequence identity of the viruses from the herd ranged between 98.7 and 100% at nucleotide level and 97.9–100% at amino acid level, and the NA sequence identity ranged between 99.2 and 100% at nucleotide level and 99–100% on the amino acid level.

3.8. Sequence identity of the herd IAV strain to the vaccine strain

Pairwise comparison of the HA sequences of the herd strain and the HA sequence of the vaccine strain (Haselünne_IDT_2617_2003 (H1N1)) revealed a 89.5–90.2% identity on nucleotide level and 90.2–91.4% on amino acid level corresponding to 48–55 amino acid differences. Eight of these amino acid differences were found in antigenic sites (Sa, Sb, Cb, Ca1 and Ca2) (Caton et al., 1982; Manicassamy et al., 2010; Rudneva et al., 2012; Yang et al., 2012) and included the following differences compared to the vaccine strain; V90A, D91N, N142H, N159K, K170G, G172K, N173G and G239E. The position of amino acid changes were numbered according to the first Methionine (H1 numbering). The pairwise comparison of the NA sequences to NA sequence of the vaccine strain (Bakum/IDT1769/2003 (H3N2)) revealed 88% sequence identity on the nucleotide level and 89% on the amino acid level.

3.9. Sequence identity among IAV strains isolated from the prolonged- and the recurrent shedders

As mentioned earlier, 18 pigs tested positive for IAV over a minimum of three sampling times, which we defined as “prolonged shedders”, and 14 pigs in total tested positive for IAV twice separated with two to three sampling times, which we defined as “recurrent shedders”. Consensus sequences of the HA gene from at least two different sampling times were successfully obtained from four recurrent shedders (N78, V81, V85 and V89) and from five prolonged shedders (N36, N38, V45, V57 and N136). For the prolonged shedders no or few (≤ 5) nucleotide changes were observed between sequences obtained from the same pig at different sampling times, and only in two of the pigs, the nucleotide difference resulted in amino acid changes (I387S, V389I for pig N38 and K40N for pig N136). Pig V45 was categorized as a “prolonged shedders” but - despite it only tested negative in week 5 - the piglet also behaved as a “recurrent shedder” as it first tested positive for IAV at week 2, 3 and 4, and then tested negative at week 5 and positive again at week 6 with a relatively low Ct value. Sequencing of samples from pig V45 revealed that at weeks 2 and 3 the HA gene was 100% identical, whereas at week 6, 12 nucleotide mutations had occurred, resulting in seven amino acid changes. The HA gene was sequenced from five pigs including V45 with recurrent infections. All recurrent shedders showed between one and 17 nucleotide substitutions between samplings, and in all pigs at least one of the nucleotide changes resulted in amino acid changes. Of the five pigs with recurrent infection, identical amino acid changes were shared at position 159, 235, 331, 387 and 389, meaning that two-three pigs showed identical mutations. Interestingly, three of these five mutations were located in HA1 which encodes the globular head of the HA protein (Steinhauer and Skehel, 2002), which is the main target for neutralizing antibodies. In addition, one of these positions (159) was located in the antigenic

site Ca2, and the same mutation K159R occurred in two different pigs (N78 and V81). The HA and NA sequences of this study are available in the NCBI Genbank with the following accession numbers: MN263260–MN263291.

4. Discussion

No adverse effects of experimental vaccination with a reduced dose of RespiPorc FLU3 were encountered during the study indicating that vaccination of very young piglets is safe despite of it being “off label” use of the vaccine. The impact of vaccination of piglets was, however, vague. Significant differences in clinical signs between the vaccinated and the control group were observed, when analyzing each sampling time separately, however, the differences were not always to the benefit of the vaccinated pigs and they were not consistent over more sampling times. In addition, no evidence of clinical protection was identified in the vaccinated group. Furthermore, the body weights at weeks 0, 3 and 6 were not significantly different between the two treatment groups, which suggested that vaccination of very young piglets did not result in a higher average daily weight gain. This was further emphasized by the fact that no differences in the prevalence of pigs with a poor body condition score between the vaccinated and the control pigs were observed.

The transfer of the piglets between different sows or pens had a negative impact on the body weight, the body score, fecal soiling and resulted in more pigs with one or more clinical signs of respiratory disease independent of IAV vaccination. This underlines that transfer of pigs between pens presents a major risk factor for compromised health and might not have the desired effect in weight gain either. Furthermore, the extensive mixing of piglets between litters and use of nursing sows in this herd present a high risk for the spread of IAV infections, and help explain the high infection level observed in the farrowing unit. Moreover, all gilts of the herd were introduced from an external source without any quarantine, thereby contributing to the maintenance of IAV circulation but also inducing a risk of novel IAV introductions.

The overall prevalence of IAV in both the vaccinated and the control group was very high and there were no overall differences between the two treatment groups. The only significant difference in the number of pigs testing positive for IAV was observed at week 4 among the weaned pigs in the nursery unit. At this time point, significant higher numbers of IAV positive pigs were identified in the control group compared to the vaccinated group. This suggested that vaccination of the piglets during week 1 decreased the number of susceptible pigs after weaning. However, the number of pigs present in the nursery at week 4 was limited, and the difference in susceptibility was not sustained in the following weeks and, as mentioned above, the overall prevalence did not vary between the two groups. In addition, the IAV positive vaccinated pigs present in the nursery at week 4 had a significantly lower average Ct value, indicating that the vaccinated pigs shed more virus. Therefore, the impact of difference observed at week 4 is inconclusive. It could be argued that the study design itself made it difficult to observe any benefit of vaccination, as vaccinated and control pigs were present in the same pen and in the same stable. However, the advantages of the chosen study design were the presence of natural transmission dynamics of IAV in the pens and a no bias in relations to the sows.

The presence of IAV was correlated to clinical signs of respiratory disease. Especially nasal discharge was strongly correlated with detection of IAV in the nasal swab, which confirms the result of a previous study investigating the impacts of IAV in the farrowing and nursery units (Ryt-Hansen et al., 2019). In addition, the presence of IAV at week 6 was also correlated with a lower weight. Together, these results emphasize that IAV indeed has a clinical and economic impact in swine herds as described in other studies (Brown et al., 1993; Er et al., 2014; Ferrari et al., 2009; Loeffen et al., 2009; Ryt-Hansen et al., 2019; Van Reeth et al., 1996).

The results from the antibody ELISA test indicated that vaccination of piglets during week 1 did not increase the prevalence of seropositive piglets in week 3 compared to unvaccinated pen-mates in that approx. 20% of the piglets from both groups were seronegative in week 3. This could be due to the vaccination procedure used in this herd. According to the Summary of Product Characteristics (SPC) for RespiPorc FLU3 the dose per pig is 2 mL administered twice. The choice to use only 0.5 mL once was made because this is the standard dosing regimen used by most Danish veterinary practitioners. This is not based on any scientific evidence, but merely a choice made empirically to reduce the price and to mitigate risk of side effects. Furthermore, no booster were given because the aim of the vaccination was to control the infection the farrowing unit. Another explanation for the lack of seroconversion could be that the piglet's immune system was not able to respond to this vaccination at such an early age. Nevertheless, previous studies on piglet vaccination against PCV2 at day 5 and *Mycoplasma hyopneumoniae* at 1 week of age (O'Neill et al., 2011; Reynolds et al., 2009; Wilson et al., 2013) suggested that the immune system is indeed able to respond efficiently to vaccination. Another more likely explanation is that the presence of maternal derived antibodies interfered with the vaccination (Loeffen et al., 2003; Renshaw, 1975), and hindered an active immune response in the piglet. However, lack of seroconversion was also seen in piglets from seronegative sows and the number of infected and vaccinated pigs from seropositive- and seronegative sows did not differ. Since no samples were taken from the colostrum or from newborn pigs, the possibility that the piglets did receive MDA despite the seronegative status of the sow cannot be confirmed.

The most likely explanation for the lack of effect of vaccination in the present herd was the fact that the piglets were infected very early in life – before a response to vaccination could be anticipated. Piglets at only four days of age became infected and the peak of infection was observed already at week 2. These results confirm the results of a previous study (Ryt-Hansen et al., 2019) and emphasize that in some herds it is extremely challenging to stimulate an antibody response before the pigs are naturally infected. Moreover, the low dosing regimen used, the inhibitory effect of MDA and strain diversity may also have an impact on the efficacy of the vaccine. Indeed, the level of homology between the vaccine strain and the circulating herd strain should be taken into consideration when evaluating the effect of vaccination. The HA of the circulating strain of the herd and the HA of the vaccine strain were of the same avian lineage, but shared only 90.2–91.4% amino acid identity. A clear correlation between amino acid homology and level of cross protection has not been finally established for swIAV, however, some of the amino acid differences between the vaccine strain and the field strain were located in specific antigenic sites, indicating that these differences might have an impact of antibody binding to the HA gene. However, further studies are needed to evaluate the impact of this finding. Moreover, the NA genes of the circulating strain and the vaccine strains were also found to vary. The internal genes of the circulating strain were, with the exception of the NS gene, of pandemic A(H1N1)pdm09 origin. However, the vaccine (RespiPorc FLU3) does not include any component of A(H1N1)pdm09 origin. A previous study has shown that some of the protective antibodies induced by whole virus inactivated vaccine are also directed against the internal genes such as the M or NP genes (Vincent et al., 2017). Moreover, the internal genes also contain important epitopes for the cellular immunity including T-cell responses (Gotch et al., 1987; Yewdell et al., 1985). Therefore, it could be speculated that the mismatch between the internal genes of the vaccine- and field strain impaired the efficacy of the vaccine; however, more studies are needed to document this rather controversial hypothesis. A modified live viral vaccine (MLV) for intra nasal use has recently been released for use in the US and has been shown to be effective when used in piglets as early as 1 day of age in the presence of MDA (Genzow et al., 2018; Vincent et al., 2012, 2007). The difference between the two vaccines is that inactivated vaccines mainly stimulates production of IgG, whereas the

MLV vaccine also induces a local IgA response (Busquets et al., 2010; Gould et al., 2017; Loeffen et al., 2003; Renegar et al., 2004; Seibert et al., 2013).

Previous studies have suggested the possibility of pigs becoming re-infected with the same strain (Chamba Pardo et al., 2019; Diaz et al., 2017a; Ryt-Hansen et al., 2019; Simon-Grifé et al., 2012) and furthermore, piglets infected with IAV in the presence of MDA have been shown to have prolonged IAV shedding time (Loeffen et al., 2003; Rose et al., 2013). The present study design included very frequent sampling, which allowed us to study this in more details. Indeed, prolonged (consecutive) shedding of IAV for 2–3 weeks were documented along with recurrent (non-consecutive) shedding indicating reinfection with the same subtype. To sustain that the pigs indeed were infected with the same strain, viral sequences were obtained from pigs considered to be prolonged shedders and from pigs considered to be re-infected. The sequence data clearly showed that only one virus strain of the H1avN2sw subtype was circulating in the herd, which is the most prevalent subtype found in Denmark (Watson et al., 2015). Furthermore, the sequences obtained from the prolonged shedders revealed very close sequence homology, which indicated that these pigs were shedding the same virus for at least 14 days. Few to no nucleotide changes were observed in these animals. Similarly, the sequences obtained from the recurrent shedders, indicated that pigs were indeed capable of being re-infected with the same strain. However, in contrast to the prolonged shedders, the HA sequence data revealed significant differences between the strain collected at the “first” infection and the “second” infection, and some of these changes were consistent between different re-infected pigs. This indicated a strong selective pressure because the amino acid changes clustered in known antigenic sites of the HA gene. This finding is very important in large swine herds chronically infected with IAV, because it will drive the evolution of viral variants towards variants that can escape the immunity against the field strain. Indeed, we have preliminary data that indicates that IAV in chronically infected herds behave similar to human seasonal viruses and undergo a stair-wise evolution and accumulate mutation in antigenic important sites (Ryt-Hansen, in preparation). These findings emphasizes that viral drift of IAV is a reality also in swine and should be considered in cases of vaccine-failure – especially in larger and unsectioned herds. Apart from genetic drift, the presence of MDA at the time of infection may also predispose for re-infection. As MDAs have been shown to weaken an active immune response to initial IAV infection, there is a risk that when the MDAs wane, the pig can become susceptible for re-infection with the same strain. Several studies have indeed shown that the presence of MDA at the time of IAV infection weakens a subsequent active immune response (Deblanc et al., 2018; Loeffen et al., 2003; Markowska-Daniel et al., 2011; Niewiesk, 2014; Rose et al., 2013). However, actual reinfection has only been shown in one study (Loeffen et al., 2003). The accumulating evidence of prolonged shedding and re-infection with the same subtype and even strain should be taken into consideration when investigating IAV dynamics and transmission in the field.

5. Conclusion

The results of this study in a single herd revealed that there was no beneficial effect of piglet vaccination with a reduced and single-shot dosing regimen of Resporc FLU3 at the time of castration. The main reason for the lack of protection was probably the early infection time combined with the reduced dosing regimen used, which made it challenging to obtain a vaccine response before infection. The results of the study confirmed that IAV infection results in clinical signs of respiratory disease and that a negative effect on body weight should be expected in herds with circulating IAV in the farrowing and nursery unit. In addition, more evidence of prolonged shedding of IAV and re-infections with the same strain were provided and the results emphasized that the dynamics of IAV in the herds are even more complex than previously

perceived. IAV is indeed a fast evolving pathogen and viral drift might be the reason for re-infections and thereby present an additional challenge for the control of the disease in the field.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2019.10.015>.

Availability of data and materials

All data is available as supplementary material online. Sequences obtained in the study can be found in NCBI GenBank.

Funding

The work was funded by SEGES Danish Pig Research Center.

Ethical statement

This study was carried out in strict accordance with the guidelines of the Good Experimental Practices (GEP) standard adopted by the European Union. All experimental procedures were conducted in accordance with the guidelines for field trials in Denmark.

Declaration of competing interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate: all samples were obtained with the farmers consent.

Acknowledgement

We thank the participating herd and veterinarian for curious co-operation and guidance.

Authors' contributions

PRH contributed to the sample collection and clinical recordings in the field. Furthermore, PRH performed the analysis of the samples, interpreted the results and drafted the manuscript. IL was the main responsible for the sample collection and the clinical recordings. Furthermore, IL performed all statistical analysis and helped interpreted the results and corrected the manuscript. CSK helped in the planning of the study, discussed the results and corrected the manuscript. JSK helped discuss the results and corrected the manuscript. LEL planned the overall study and helped with interpretation of the results and corrected and approved the final draft of the manuscript.

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